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14. ABSTRACT

The most significant finding in this research period has been that making breast cancer cells folate deficient is difficult to do without killing the cells. It is much easier to target folate and one carbon metabolism in different ways such as inhibiting key enzymes with either miRNA or drugs. This is the rationale for why we have switched to making tetracycline inducible stably transduced cell lines that express miRNA against either dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), s-adensoylhomocysteinase (AHCY) or DNA methyltransferase 1 (DNMT1). We have succeeded in expressing constructs containing miRNA for DHFR and AHCY in both MCF7 and MDA-MB-231 cells. The next steps involve selecting clones with non-leaky tetracycline repressor protein systems so that we can create the final tetracycline inducible, stably transduced cell lines. Once this has been accomplished we will begin to characterize the changes that occur to breast cancer cell growth and function when inhibition of either DHFR, AHCY, MTHFR or DNMT1 is induced.

15. SUBJECT TERMS
Folate, breast cancer, dihydrofolate reductase, DNA methyltransferase, methylenetetrahydrofolate reductase, S-adenosylhomocysteinase

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Introduction: This training grant focuses on one carbon and folate metabolism and the effects of perturbing one carbon metabolism on epigenetic regulation and breast cancer cell phenotype and function. The objective of the project is to understand the relationships between folate, one carbon metabolism, DNA methylation and gene expression within the context of breast cancer. Our hypothesis is that the inhibition of key enzymes in one carbon metabolism: dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), S-adenosylhomocysteinase (AHCY) and DNA methyltransferase 1 (DNMT1) by miRNA will decrease breast cancer cell growth, modulate cell migration, and decrease the ability of the cell to survive anoikis by increasing expression of hypermethylated genes including transcription factors.

Body:

Original Tasks
To characterize the effects on global and gene specific methylation and gene expression in human breast carcinoma (MCF7, MDA-MB231) and mouse mammary tumor cells (Met-1, DB-7) of the following:

a. Folate deficiency
b. 5-aza-2'-deoxycytidine (ADC) exposure
c. siRNA directed against DNA methyltransferase 1 (DNMT1)
d. siRNA directed against MBD2 protein

Changes that have been made and the reasons for changes are as follows:

1. **Work in four cell lines: MCF7 and MDA-MB-231 (human) and Met1 and DB-7 (mouse).** *We are currently working in the two human cell lines MCF7 and MDA-MB-231.* Making cells folate deficient proved to be difficult (see #2 for more information), so we decided to make tetracycline inducible stable knockdowns targeting four key enzymes in one carbon metabolism instead. The amount of work involved in doing this is much greater than that originally proposed, and therefore we decided to concentrate on making the stably transduced cell lines using the human cell lines first, with the thought that we could repeat our successes in the mouse cell lines if time and funding allowed for it. To date we have successfully transduced both MCF7 and MDA-MB-231 cells with lentivirus plasmids containing miRNA against DHFR and AHCY.
2. **Test effects of folate deficiency on global and gene specific DNA methylation and gene expression.** *Achieving sufficient folate deficiency was difficult without affecting cell growth and replication.* We decided to take a different approach and inhibit 4 enzymes involved in one carbon and folate metabolism specifically dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), DNA methyltransferase 1 (DNMT1) and S-adenosylhomocysteinase

(AHCY). We are in the process of creating tetracycline inducible, stably transduced cell lines that express miRNA against each of the four enzymes when the cell line is treated with tetracycline. Once the cell lines are made we will study the effects of depletion of protein expression on breast cancer cell growth, apoptosis, replication, migration, ability to survive anoikis, DNA methylation (global and gene specific) and concentrations of one carbon metabolites (S-adenosylmethionine, S-adenosylhomocysteine and homocysteine) (Table 1).

3. **Effect of DNMT1 inhibition by 5'-aza deoxycytidine (ADC) on global and gene specific DNA methylation and gene expression.** *We plan to inhibit DNMT1 with both ADC and lentiviral mediated transduction of miRNA against DNMT1.*
4. **Effect of MBD2 inhibition on gene expression.** Since we are no longer testing the effects of folate deficiency we decided to focus more closely on inhibiting enzymes more closely related to folate and one carbon metabolism i.e. DHFR, MTHFR and AHCY.

Once the tetracycline inducible stably transduced cell lines are made, we will perform the assays outlined in Table 1 to assess changes in breast cancer cell function and phenotype.

Table 1: Assays to assess breast cancer cell function and phenotype after inhibition of either DHFR, MTHFR, AHCY or DNMT.

Measured Endpoint	Assay
Cell proliferation	Trypan blue exclusion assay and MTT assay
Cell apoptosis	TUNEL assay and caspase 3 staining
Cell migration	Scratch assay
Anoikis	Propidium iodide staining followed by flow cytometry
One carbon metabolite levels: [S-adenosylmethionine], [S-adenosylhomocysteine] and [Homocysteine]	High performance liquid chromatography (HPLC)
Differential gene expression	mRNA microarray
Gene-specific promoter methylation	Methylation specific PCR or pyrosequencing

Key Research Accomplishments:

1. Completed lentiviral constructs containing two independently designed miRNA sequences for each of the four proteins. This is to ensure that if the first miRNA sequence does not inhibit expression of the protein we will have a second construct readily available to try.
2. Demonstrated that the lentivirus containing DHFR miRNA and AHCY miRNA constructs has been successfully incorporated into both the MCF7 and MDA-MB-231 cell lines.
3. Increased confidence and proficiency by the PI in lentiviral construct development, bacterial transformation, transfection and transduction of MCF7 and MDA-MB-231 cells, HPLC assay development, western blots, and enzyme activity assays.
4. Increased enthusiasm and motivation to pursue research in breast cancer after attending the Era of Hope conference 2011.

Reportable Outcomes:

Abstract published and poster presentation at the Era of Hope conference 2011.

Conclusion: Several changes have been made to the original research plan. Mainly the development of tetracycline inducible stably transduced MCF7 and MDA-MB-231 cells expressing miRNA against one of the following enzymes: dihydrofolate reductase, methylenetetrahydrofolate reductase, DNA methyltransferase 1 and S-adenosylhomocysteinase. We decided to perturb one carbon metabolism using this approach because the original approach of making cells folate deficient proved too difficult to do without killing the cells. We are focusing on making the stably cell lines from two parent cell lines of human origin. If time and funding permit we will repeat the process in the mouse cell lines listed in the original research plan (DB7 and Met 1).

Appendix

Please see PDF attachment to email for abstract submitted and published at the Era of Hope conference 2011.

Poster P11-8

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EPIGENETIC MECHANISMS OF FOLATE NUTRITION IN BREAST CANCER

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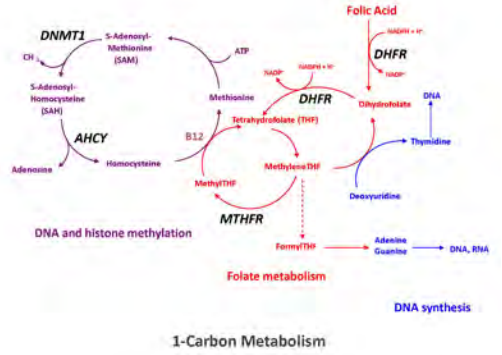

One Carbon Metabolism

In 1998 the U.S. government implemented mandatory fortification of cereal grain products with folic acid to decrease the incidence of neural tube defects (NTDs). While this action was successful in lowering NTD incidence, recent epidemiological studies are highlighting a possible association between excess folic acid and cancer incidence. Current research on the effects of excess folic acid on breast cancer initiation and progression are inconclusive.

DNA methylation is a form of epigenetic regulation that results in chromatin remodeling and ultimately gene silencing. It is essential for cellular differentiation and function. Aberrant DNA methylation is a characteristic of cancer cells, including mammary tumors. The B vitamin folate is required for the synthesis of purines, thymidine, and S-adenosylmethionine (SAM), the methyl donor for DNA methylation. DNA methyltransferase 1 maintains methylation patterns and catalyzes the transfer of the methyl group from SAM to a cytosine residue.

Our objective is to understand the relationships between folate, one-carbon metabolism, DNA methylation, and gene expression within the context of breast cancer. We hypothesize that modulation of folic acid levels or inhibition of key enzymes in one-carbon metabolism (dihydrofolate reductase [DHFR], methylenetetrahydrofolate reductase [MTHFR], DNA methyltransferase 1 [DNMT1], or adenosylhomocysteinase [AHCY]) will affect breast cancer cell growth.

Working with two parent breast cancer cell lines, MCF7 and MDA MB 231, we have developed tetracycline-inducible, stably transfected cell lines that have one of the enzymes listed (DHFR, MTHFR, DNMT1, or AHCY) knocked down. We will compare the effects of knocking down enzyme expression in this way to the effects of treating breast cancer cells with methotrexate or excess folic acid. We will measure changes in cell growth, migration, survival of anoikis, global and gene-specific DNA methylation changes, as well as one-carbon metabolite levels.

Folate metabolism is important in DNA synthesis and methylation. The U.S. population is frequently exposed to twice or more the recommended dietary allowance of folic acid, and there is a need to determine the effects of excess folic acid on cancer cell proliferation, which can serve as a surrogate of cancer progression.

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